AD_____

Award Number: DAMD17-00-1-0457

TITLE: Involvement of BRCA2 Repeats in RAD51 Mediated DNA Repair

PRINCIPAL INVESTIGATOR: Qing Zhong, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas Health Science Center at San Antonio
San Antonio, Texas 78229-3900

REPORT DATE: December 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 2001	Annual Summary (1 Dec 00 - 30 Nov 01)

4. TITLE AND SUBTITLE
Involvement of BRCA2 Repeats in RAD51 Mediated DNA Repair

5. FUNDING NUMBERS
DAMD17-00-1-0457

6. AUTHOR(S)
Qing Zhong, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas Health Science Center at San Antonio
San Antonio, Texas 78229-3900

E-Mail: zhong@uthscsa.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The overall goal of my grant proposal is to study how BRCA2 is involved in repairing DNA damage. A better understanding of BRCA2's role in breast cancer could prevent cancer formation and reduce resistance to cancer therapies. To test our hypothesis, we proposed to accomplish the following specific aims. Aim1. To test the importance of BRC repeats in BRCA2 for binding to Rad51 in response to DNA damage. Aim2. To determine the critical residues in the BRC repeats of BRCA2 and the significance of these residues for BRCA2/Rad51 interactions. We screened for BRC repeat mutant that fail to bind to RAD51 through random mutagenesis, and one of the mutants competitively inhibited BRC-RAD51 binding was specifically identified and used for sebsequent analysis. We established cell lines tetracycline-controlled inducibly expressing of wild -type BRC repeat-GFP fusion peptides or mutant BRC repeat-GFP fusion peptide in MCF7 cells. We found that endogenous BRCA2-RAD51 binding was abolished by wild-type BRC repeat but not mutant BRC repeats. The characterization of BRC repeat inducible expression cell lines in DNA damage response and DNA repair will represent an important step toward our goal in understanding the significance of the interaction between BRCA2 and RAD51 repair complex.

14. SUBJECT TERMS
Tumor suppressor gene, BRCA2, DNA repair

15. NUMBER OF PAGES
14

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover..... 1

SF 298..... 2

Table of contents..... 3

Introduction..... 4

Body..... 5-7

Key Research Accomplishments..... 8

Conclusions..... 9

References..... 10

Appendices..... 12-14

Introduction

Mutations in the BRCA2 gene have been found in many familial breast cancers (Miki et al., 1996, Easton et al., 1993, and Couch et al., 1996). Cells deficient in BRCA2 or containing BRCA2 mutations lead to various cellular abnormalities including increased sensitivity to DNA damage by genotoxic agents, accumulation of DNA double-strand breaks, chromosome abnormalities, and changes in cell cycle check point and apoptotic responses (Ludwig et al., 1997, Sharan et al., 1997, and Suzuki et al., 1997, Patel et al., 1998). It was proposed that BRCA2 could be involved in DNA repair. This hypothesis was further supported by evidence suggesting that BRCA2 interacts with Rad51, a protein involved in DNA recombination repair (Sharan et al., 1997). BRCA2 binds Rad51 through six highly conserved BRC repeats (Chen et al., 1998); however, the biological role associated with these interactions is yet to be determined. In this proposal, we plan to test the significance of these interactions in cellular function and DNA repair by: 1) expression of wild-type and mutated BRC repeats in cells treated with DNA damaging agents 2) expression of BRCA2 containing single point mutations in BRC repeats in cells treated with DNA damaging agents 3) expression of short peptides mimicking wild-type BRC repeats to sensitize tumors in nude mice to DNA damaging agents . Determining the precise role of BRCA2 in Rad51 mediated DNA repair could be an important step in cancer prevention and in combating resistance to radiation therapy.

Body

We have made the following progress on testing the novel idea that BRC repeat play an important role in DNA double-strand break repair and damage response.

1) To determine the critical residues in the BRC repeats of BRCA2 and the significance of these residues for BRCA2/Rad51 interactions.

1. Identification of mutations in the first and fourth BRC repeats of BRCA2 that result abolish Rad51 binding.

To systematically address the biological consequence of the interaction between BRCA2 and Rad51, amino acid residues of the first BRC repeat, BRC1, that are critical for Rad51-binding were first identified. BRC1 was subjected to biased PCR mutagenesis (Shan et al., 1996), and the mutated cDNAs were translationally fused to the GAL4 DNA-binding domain in the yeast vector, pAS1 (Durfee et al., 1993), to generate a library of $2x10^6$ individual clones referred to as pAS/BRC1-ML. A reverse two-hybrid screen with negative selection was used to isolate clones that fail to bind Rad51 as described previously (Vidal et al., 1996). Several mutations in BRC1 were identified that significantly reduced Rad51-binding in a yeast two-hybrid assay (Table 1). BRC1-M1 is a mutation that changes a conserved threonine residue to alanine. BRC1-M2 and -M3 are changes in non-consensus amino acids, and BRC1-M4 carries a double mutation at the two C-terminal BRC1 residues, the last residue of which is conserved. Interestingly, a familial mutation, G1529R, has been previously found in BRC4 (Breast Cancer Information Core). Specific Rad51 binding activity by BRC4 was also tested and found to be approximately three times stronger than BRC1 (Table 1). Two BRC4 mutations, BRC4-M5, an analogous mutation to BRC1-M1, in which the conserved threonine at the third position is changed to an alanine, and BRC4-M6 which contains the G1529R mutation, were constructed and have reduced Rad51-binding (Table 1). These results suggest that, despite their sequence conservation, the ability of BRC repeats to bind Rad51 varies, and is dependent on specific residues.

2. Conditional expression of wild-type and mutated GFP-BRC4 fusions in MCF7 cells

To determine the functional importance of the interactions between the BRC repeats of BRCA2 and Rad51, cells that conditionally express BRC repeats fused to the green fluorescent protein (GFP) were generated. A GFP-BRC4 repeat fusion protein also containing a nuclear localization signal and a myc epitope (Fig 1A) was subcloned into an expression plasmid (pUHD10-3) driven by the core cytomegalovirus (CMV) promoter linked to a tet operator heptad (pUHD10-3/GFP-BRC). A similar expression plasmid for BRC4-M5 was also made. These two plasmids were separately co-transfected into MCF-7 cells with pCHTV, bearing a hygromycin-resistance gene and a CMV-controlled tetracycline repressor-VP16 fusion transcription unit (Chen et al., 1996). Hygro-resistant clones were subsequently isolated.

Two stable lines of MCF-7 cells, WT-8 and MT-11, that conditionally express wild-type and BRC4-M5 mutant, respectively, were established (Fig. 1A). Tetracycline-responsive expression of the GFP-BRC4 fusion proteins in these two lines was clearly demonstrated by immunoprecipitation with α-myc antibodies and immunoblotting with α-GFP (Fig. 1B, top panel compare lanes 2 and 4 with 1 and 3).

3. Over-expression of wild-type, but not mutated, BRC4 repeats diminished the interaction between BRCA2 and Rad51

Rad51 is detected in the immunoprecipitates of wild-type, but not GFP-BRC4-M5 (Fig. 14B, compare lane 4 with 2), indicating that the GFP fusion with wild-type BRC4 binds to Rad51 in cells. Importantly, expression of wild-type, but not the BRC4-M5 mutant, significantly reduced the amount of BRCA2 in the Rad51 immunoprecipitates, and, in the reciprocal experiment, reduced the amount of Rad51 in the BRCA2 immunoprecipitates (Fig. 2, compare lane 4 with lane 2). These data strongly suggest that over expression of the wild-type, but not mutated, BRC4 repeat effectively disrupts the interaction between BRCA2 and Rad51.

The establishment of these inducible clones will provide an extremely useful tool for us to further study the functional significance of the interaction between BRCA2 and RAD51 in DNA damage repair and response in vivo.

2) *To test the importance of BRC repeats in BRCA2 for binding to Rad51 in response to DNA damage.*

To accomplish this task, we are in the process to determine the cellular activities including sensivity to various DNA damage agents treatment, radiation-induced focus formation, and DNA damage cell cycle checkpoint in these inducible clones in which BRCA2-Rad51 interaction are specifically interrupted or not.

1. Determine if Over-expression of wild-type BRC4 repeats associates with hyper-sensitivity to various DNA damaging agents

Since Rad51 plays a critical role in the repair of DNA double-strand breaks by homologous recombination (Shinohara et al., 1992), disruption of the interaction between BRCA2 and Rad51 may have an adverse effect on the ability of cells to respond to DNA damage. To test this possibility, both WT-8 and MT-11 cells cultured either with or without tetracycline, will be mock-exposed or exposed to various DNA damaging agents treatments including ionizing radiation, UV , MMS, and MMC, and cell survival will be determined by a clonogenic assay. We expect induced expression of wild-type BRC4 repeats (WT-8, - Tet) will be associated with a significantly reduced cell survival rate when compared to uninduced expression of wild-type (WT-8, + Tet) or to either induced (MT-11, - Tet) or uninduced (MT-11, + Tet) expression of mutated BRC4.

2. To test for BRC's effect in modulating the focus formation of Rad51 following ionizing radiation

To further explore the DNA damage response of the WT8 and MT11 cell lines, the appearance of radiation-induced Rad51-containing foci will be examined. We expect that under uninduced conditions (+Tet), both clonal lines will form Rad51 foci after γ-irradiation as we previously reported in T24 cells (Zhong et al., 1999). However, WT-8 cells induced to express wild-type GFP-BRC4 repeat will exhibite a reduction in the appearance of Rad51 foci compared to cells induced to express the GFP-BRC4-M5 mutated repeat. These data will provide insight information if the interaction between BRCA2 and Rad51 is crucial for the formation of Rad51 repair foci and, furthermore, that exogenous expression of BRC repeats can interfere with this activity.

3. To test the effect of over-expression of wild-type BRC4 repeats in MCF7 cells on G1/S and G2/M checkpoint

Increased sensitivity to ionizing radiation may result from defects in the DNA repair machinery or in the molecules essential for cell cycle checkpoint control. When normal mouse embryo fibroblasts (MEF) are exposed to γ-irradiation, their transit through the cell cycle is arrested at one of two points (Kuerbitz et al., 1992, and Xu et al., 1999). The G1/S checkpoint, which is dependent upon p53 and p21 (Kastan et al., 1992, Lu and Lane, 1993, Brugarolas and Jacks, 1997, and Deng et al., 1995), prevents the replication of damaged DNA. The G2/M checkpoint prevents segregation of damaged chromosome (Elledge, 1996). To test for a potential role of BRCA2/Rad51 interactions in DNA damage-induced cell cycle checkpoint control, cells expressing the GFP-BRC4 repeats will assayed for G1/S and G2/M checkpoint integrity in response to γ-irradiation.

4. To test small BRC repeat peptides in vivo for their ability to overcome tumor resistance to DNA damaging agents

Once the cellular effect of BRC repeat interference on BRCA2-RAD51 interaction is determined, we shall proceed to tumor resistance experiments as proposed.

Key Research Accomplishments

1. Mutations in the BRC repeats of BRCA2 that result abolish Rad51 binding are identified.
2. Cell clones that conditional expressing wild-type and mutated GFP-BRC4 fusions in MCF7 cells are established.
3. Over-expression of wild-type, but not mutated, BRC4 repeats diminished the interaction between BRCA2 and Rad51 in vivo.

Conclusions

Since the beginning of the grant funding, we have successfully screened out BRC repeat mutants that could not bind to RAD51 and established cell lines inducibly expressing wild-type and mutant BRC repeat. Through a reverse two-hybrid screen to isolate clones that fail to bind Rad51. Several mutations in BRC1 and BRC4 were identified that significantly reduced Rad51-binding. To determine the functional importance of the interactions between the BRC repeats of BRCA2 and Rad51, cells that conditionally express wild-type or mutant BRC repeats fused to the green fluorescent protein (GFP) were generated. We demonstrated that wild-type, but not mutated, BRC4 repeat effectively disrupts the interaction between BRCA2 and Rad51 in vivo. We are in the process of examining the cellular activities including sensivity to various DNA damage agents treatment, radiation-induced focus formation, and DNA damage cell cycle checkpoint in these inducible clones. These are straightforward experiments, we do not anticipate any difficulties in these experiments.

References

BIC. Breast Cancer Information Core: www.nchgr.nih.gov

Brugarolas, J., and Jacks, T. (1997). Double indemnity: p53, BRCA and cancer. p53 mutation partially rescues developmental arrest in Brca1 and Brca2 null mice, suggesting a role for familial breast cancer genes in DNA damage repair. Nature Medicine *3*, 721-2.

Chen, P.-L., Riley, D. J., Chen, Y., and Lee, W.-H. (1996). Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. Genes Dev. *10*, 2794-2804.

Chen, P. L., Chen, C. F., Chen, Y., Xiao, J., Sharp, Z. D., and Lee, W. H. (1998). The BRC repeats in BRCA2 are critical for RAD51 binding and resistance to methyl methanesulfonate treatment. Proc Natl Acad Sci U S A *95*, 5287-5292.

Couch, F. J., Farid, L. M., Deshano, M. L., Tavtigian, S. V., Calzone, K., Campeau, L., Peng, Y., Bogden, B., Chen, Q., Neuhausen, S., Shattuck-Eidens, D., Godwin, A. K., Daly, M., Radford, D. M., Sedlacek, S., Rommens, J., Simard, J., Garber, J., Merajver, S., and Weber, B. L. (1996). *BRCA2* germline mutations in male breast cancer cases and breast cancer families. Nature Genetics *13*, 123-125.

Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leder, P. (1995). Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell *82*, 675-84.

Durfee, T., Becherer, K., Chen, P.-L., Yeh, S.-H., Yang, Y., Kilburn, A. E., Lee, W.-H., and Elledge, S. J. (1993). The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes Dev. *7*, 555-569.

Elledge, S. J. (1996). Cell cycle checkpoints: preventing an identity crisis. Science *274*, 1664-72.

Easton, D. F., Bishop, D. T., Ford, D., and Crockford, G. P. (1993). Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. The Breast Cancer Linkage Consortium. American Journal of Human Genetics *52*, 678-701.

Kastan, M. B., Zhan, Q., el-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Vogelstein, B., and Fornace, A. J., Jr. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell *71*, 587-597.

Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V., and Kastan, M. B. (1992). Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proceedings of the National Academy of Sciences of the United States of America *89*, 7491-5.

Lu, X., and Lane, D. P. (1993). Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? Cell *75*, 765-78.

Ludwig, T., Chapman, D. L., Papaioannou, V. E., and Efstratiadis, A. (1997). Targeted Mutations Of Breast Cancer Susceptibility Gene Homologs In Mice: Lethal Phenotypes Of Brca1, Brca2, Brca1/Brca2, Brca1/P53, And Brca2/P53 Nullizygous Embryos. Genes and Development *11*, 1226-1241.

Miki, Y., Katagiri, T., Kasumi, F., Yoshimoto, T., and Nakamura, Y. (1996). Mutation analysis in the BRCA2 gene in primary breast cancers. Nature Genetics *13*, 245-247.
Patel, K. J., Vu, V. P., Lee, H., Corcoran, A., Thistlethwaite, F. C., Evans, M. J., Colledge, W. H., Friedman, L. S., Ponder, B. A., and Venkitaraman, A. R. (1998). Involvement of Brca2 in DNA repair. Mol Cell *1*, 347-57.

Shan, B., Durfee, T., and Lee, W. H. (1996). Disruption of RB/E2F-1 interaction by single point mutations in E2F-1 enhances S-phase entry and apoptosis. Proceedings of the National Academy of Sciences of the United States of America *93*, 679-684.

Sharan, S. K., Morimatsu, M., Albrecht, U., Lim, D. S., Regel, E., Dinh, C., Sands, A., Eichele, G., Hasty, P., and Bradley, A. (1997). Embryonic Lethality And Radiation Hypersensitivity Mediated By Rad51 In Mice Lacking *Brca2*. Nature *386*, 804-810.
Suzuki, A., de la Pompa, J. L., Hakem, R., Elia, A., Yoshida, R., Mo, R., Nishina, H., Chuang, T., Wakeham, A., Itie, A., Koo, W., Billia, P., Ho, A., Fukumoto, M., Hui, C. C., and Mak, T. W. (1997). *Brca2* Is Required For Embryonic Cellular Proliferation In The Mouse. Genes and Development *11*, 1242-1252.

Patel, K. J., Vu, V. P., Lee, H., Corcoran, A., Thistlethwaite, F. C., Evans, M. J., Colledge, W. H., Friedman, L. S., Ponder, B. A., and Venkitaraman, A. R. (1998). Involvement of Brca2 in DNA repair. Mol Cell *1*, 347-57.

Vidal, M., Brachmann, R. K., Fattaey, A., Harlow, E., and Boeke, J. D. (1996). Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein and DNA-protein interactions [see comments]. Proc Natl Acad Sci.U S A *93*, 10315-20.

Xu, X., Weaver, Z., Linke, S. P., Li, C., Gotay, J., Wang, X. W., Harris, C. C., Ried, T., and Deng, C. X. (1999). Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells. Mol Cell *3*, 389-95.

Zhong, Q., Chen, C. F., Li, S., Chen, Y., Wang, C. C., Xiao, J., Chen, P. L., Sharp, Z. D., and Lee, W. H. (1999). Association of BRCA1 with the hRad50-hMre11-p95 complex and the DNA damage response. Science *285*, 747-50.

Appendices

Table 1:Mutations of BRC repeats inactivate its Rad51 binding activity

BRC Consensus	I I FXTASGKX X SXXXLXKXXXX X V V F E	Realtive β-gal activity
BRC1	FRTASNKEIKLSEHNIKKSKMFFKD	7
BRC1-M1	A	<1
BRC1-M2	G	<1
BRC1-M3	S	<1
BRC1-M4	RG	<1
BRC4	FHTASGKKVKIAKESLDKVKNLFDE	22
BRC4-M5	A	<1
BRC4-M6	R	4
vector		1

Table 1: Identification of mutations in the first and fourth BRC repeats of BRCA2 that disrupt binding to Rad51.A randomly mutagenized pool of cDNAs encoding BRC1 repeats (amino acid 1009 to 1032) was cloned into the pAS1 vector and co-transformed along with pGAD-Rad51 into Mav203 cells. Four clones with DNA inserts that showed no detectable β-galactosidase activity in a yeast two-hybrid assay were isolated. Mutations with amino acid changes resulted from single nucleotide changes. Rad51-binding activity using the BRC4 in pAS1 vector was also tested in the assay. Note that this repeat has 3 fold higher activity compared to the BRC1 repeat. The T to A mutation identified above in the BRC1 repeat that abrogates Rad51 binding, and a familial mutation identified in BRC4 (G1529R) was introduced into the BRC4 and tested in the yeast-two hybrid assay. Both of the mutations significantly reduced, but the G to R mutation did not completely eliminate, Rad51-binding in this assay.

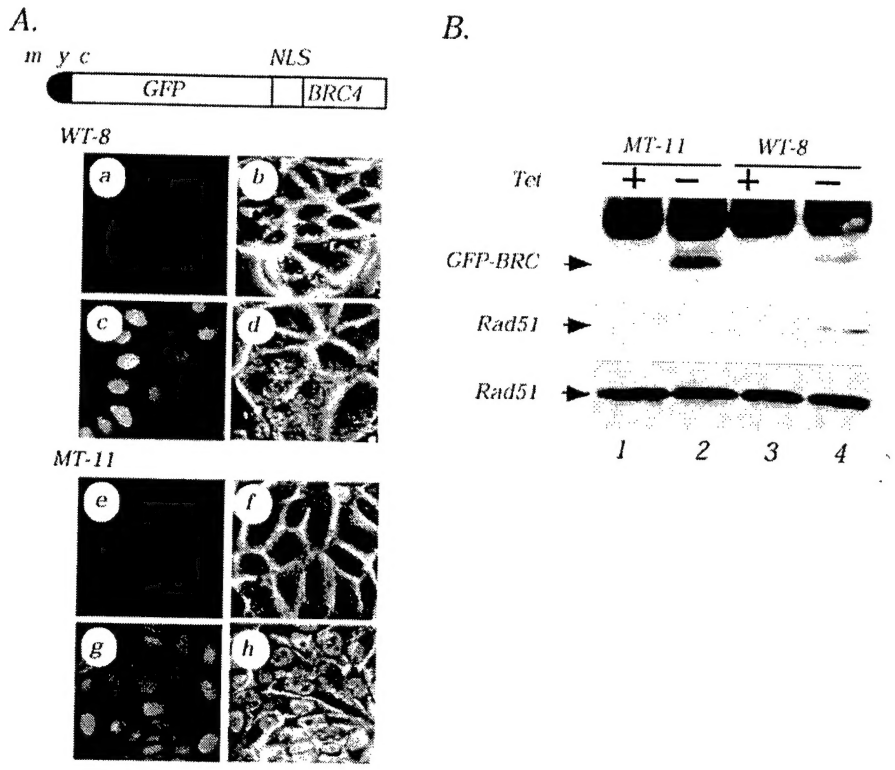

Figure 1. Conditional expression of wild-type and mutated GFP-BRC4 repeat in MCF7 cells *A*. Schematic drawing of the GFP-BRC4 fusion containing a BRC4 cDNA fragment translationally fused to a myc epitope-GFP-NLS cassette in a modified pUHD10-3 plasmid, pUHD10-3/GFP-BRC (Top panel). Cell clones expressing the wild-type GFP-BRC4 (WT-8) (a-d) and mutated GFP-BRC4-M5 (MT-11) (e-h), were visualized by GFP autofluorescence (a, c, e, and g) after incubation in the presence (a, b, e, and f) or absence (c, d, g, and h) of tetracycline (Tet). Fluorescence overlaid with phase-contrast images (b, d, f, and h) show nuclear localization of these fusion proteins. *B*. Co-immunoprecipitation of GFP-BRC4 with Rad51 in cells. Cells expressing wild-type or mutated GFP-BRC4 were immunoprecipitated with α-myc antibody (top two panels), immunoblotted with either α-GFP to detect the GFP-BRC4 fusions or α-Rad51 (indicated in the left margin). Immunoprecipitation and Western blotting with α-Rad51 antibody (bottom panel) determined the relative levels of endogenous Rad51.

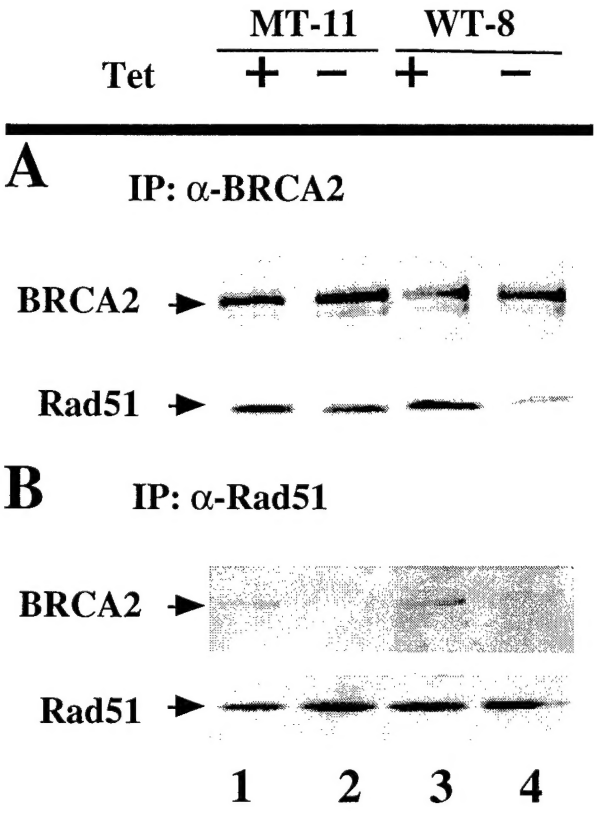

Figure 2. Expression of wild-type GFP-BRC4 fusion inhibits the interaction between BRCA2 and Rad51 Expression of wild-type GFP-BRC4 reduces the complex formation between BRCA2 and Rad51. Inducible of wild-type (lane3, and 4) or mutated GFP-BRC4 (lane1, and 2) cell line were examined. Thirty-six hours after induction of GFP-BRC4 expression (lane 1 and 3 indicated un-induced), cell lysates were co-immunoprecipitated with either α-BRCA2 (top panel) or α-Rad51 antibody (bottom panel). The resulting immune complexes were analyzed by immunoblot analysis with either α-BRCA2 or α-Rad51 antibody, indicated on the left margin.